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ROLE OF MAP KINASE IN MEDIATING THE EFFECTS OF VITAMIN D_3 METABOLITES ON GROWTH PLATE CHONDROCYTES

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ROLE OF MAP KINASE IN MEDIATING THE EFFECTS OF VITAMIN-D_3 METABOLITES ON GROWTH PLATE CHONDROCYTES

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ROLE OF MAP KINASE IN MEDIATING THE EFFECTS OF VITAMIN D_3 METABOLITES ON GROWTH PLATE CHONDROCYTES

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Endochondral bone formation involves a developmental cascade of cell differentiation and maturation, which culminates in mineralization of the extracellular matrix by chondrocytes. The process is required for normal long bone growth and in

certain kinds of bone repair. Vitamin D_3 plays an important regulatory role in chondrocyte differentiation and maturation and therefore is essential for proper endochondral ossification. The vitamin D_3 family contains two metabolites, $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$, which have been shown to be active in growth plate cartilage. The actions of these vitamin D_3 metabolites are cell maturation-dependent, with $1\alpha,25(OH)_2D_3$ primarily affecting growth zone chondrocytes and $24R,25(OH)_2D_3$ primarily affecting resting zone chondrocytes. Regulation of endochondral bone formation involves both of these vitamin D_3 metabolites.

Previous studies have demonstrated that treatment of growth zone chondrocytes with $1\alpha,25(OH)_2D_3$ increases alkaline phosphatase and phospholipase A_2 specific activities, as well as prostaglandin E_2 production. By contrast, $24R,25(OH)_2D_3$ treatment of resting zone chondrocytes increases alkaline phosphatase specific activity, but decreases phospholipase A_2 specific activity, and subsequent prostaglandin E_2 production. Studies have also shown that $1\alpha,25(OH)_2D_3$ stimulates protein kinase C alpha via activation of phosphatidylinositol-specific phospholipase C, resulting in the formation of diacylglycerol and inositol-1,4,5-trisphosphate. In contrast, $24R,25(OH)_2D_3$ increases PKC via phospholipase D-dependent production of diacylglycerol.

Inhibition of protein kinase C blocks many of the physiological responses of growth zone cells to $1\alpha,25(OH)_2D_3$ and of resting zone cells to $24R,25(OH)_2D_3$, suggesting that the pathways initiated by activation of the enzyme may have genomic effects. This is also true for protein kinase A-dependent signaling. Both signaling pathways converge at mitogen-activated protein (MAP) kinase, which then pdosphorylates transcription factors that modulate gene expression.

The current study is based on the hypothesis that MAP kinase mediates at least some of the effects of $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ in a cell maturation-dependent and vitamin D_3 metabolite-specific manner. To prove or disprove the hypothesis, the aims of the present study were: 1. To determine if MAP kinase is expressed in growth zone and resting zone chondrocytes; 2. To determine if $1\alpha,25(OH)_2D_3$ regulates MAP kinase activity in growth zone chondrocytes, and if $24R,25(OH)_2D_3$ regulates MAP kinase activity in resting zone chondrocytes; 3. To determine if MAP kinase mediates the effect of $1\alpha,25(OH)_2D_3$ in growth zone chondrocytes, and $24R,25\text{-}(OH)_2D_3$ in resting zone chondrocytes; and 4. To determine which signaling pathways are used by vitamin D_3 metabolites to enter into the MAP kinase pathway in growth zone and resting zone chondrocytes.

Resting zone and growth zone chondrocytes, representing two distinct stages of cell maturation, were isolated from rat costochondral cartilage using a well-characterized cell culture system. Expression of extracellular signal-regulated protein kinase (ERK) 1/2 family of MAP kinase was evaluated by reverse transcription and polymerase chain reaction, and MAP kinase activity in both cell types was determined after treatment with 10^{-9} to 10^{-7} M $24R,25(OH)_2D_3$ or 10^{-10} to 10^{-8} M $1\alpha,25(OH)_2D_3$. To determine if the MAP kinase activation involved protein kinase C, specific inhibitors of protein kinase C (chelerythrine and H-7) were used. Similarly, the role of protein kinase A was evaluated by using the inhibitor H-89, which is specific for this enzyme. The involvement of phospholipase C and phospholipase D in mediating the effects of the two vitamin D_3 metabolites on MAP kinase were assessed by using inhibitors specific for

phosphatidylinositol-specific phospholipase C (U73122) and phospholipase D (wortmannin).

MAP kinase activity was regulated by $1\alpha,25(OH)_2D_3$ in growth zone and by $24R,25(OH)_2D_3$ in resting zone chondrocytes, in both cases via a protein kinase C-dependent mechanism. The effect of vitamin D_3 metabolites on MAP kinase activity involved phospholipase C in growth zone cells, but not in resting zone cells, and phospholipase D in resting zone cells, but not in growth zone cells. Protein kinase A was not involved in the activation of ERK 1/2 in either group.

These results support the hypothesis that MAP kinase is expressed in growth zone and resting zone chondrocytes and mediates at least some of the effects of $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ in a cell maturation-dependent and vitamin D_3 metabolite-specific manner. $1\alpha,25(OH)_2D_3$ increases MAP kinase activity via phospholipase C and increased prostaglandin production, while $24R,25(OH)_2D_3$ increases MAP kinase activity via phospholipase D and decreased prostaglandin production. This study provides another step toward understanding the mechanisms of signal transduction in growth plate chondrocytes, and may lead to new clinical applications involving bone and cartilage formation, repair, and regeneration. It may be possible to develop periodontal therapeutic modalities that target the vitamin D_3 membrane receptors to facilitate alveolar bone repair, regeneration, or implant osseointegration.

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INTRODUCTION

Endochondral ossification involves a developmental cascade of chondrocyte differentiation and maturation, which culminates in mineralization of the cartilage extracellular matrix. The calcified cartilage then serves as a scaffold for bone maturation. The process is required for normal long bone growth and in certain kinds of bone repair (Boyan et al., 1992). This complex sequence of events is regulated by hormones and local factors. Any disruption of this process can result in improper long bone formation and/or poor bone repair. Vitamin D_3 plays an important role in regulating chondrocyte differentiation and maturation (Atkin et al., 1985; Binderman et al., 1984) and is therefore essential for proper endochondral ossification (Boskey, 1981; Anderson, 1969).

Vitamin D_3 has been associated with cartilage and bone metabolism for many years. The vitamin D_3 family contains two metabolites, $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$, which are active in growth plate cartilage. The primary function of $1\alpha,25(OH)_2D_3$ is to promote Ca^{2+} transport (Lieberherr et al., 1989) and maintain extracellular Ca^{2+} levels, while the primary function of $24R,25(OH)_2D_3$ appears to be inhibition of chondrocyte proliferation and promotion of early differentiation events (de Boland et al., 1992). Receptors for both metabolites have been identified in cartilage (Balmain et al., 1993; Fine et al., 1985; Corvol et al., 1980) and a number of studies have examined both their genomic and nongenomic effects (Boyan et al., 1994; Swain et al., 1993; Sylvia et al., 1993; Boskey et al., 1992; Schwartz et al., 1991; Gerstenfeld et al., 1990; Schwartz et al., 1990; Hale et al., 1986).

To explore the mechanism of vitamin D_3 action, a cell culture model was developed using rat costochondral chondrocytes (Boyan et al., 1994; Boyan et al., 1992; Boyan et al., 1988). By taking advantage of the fact that rat costochondral cartilage is organized into

distinct zones, nearly homogenous cell populations from the resting zone (reserve zone) and growth zone (prehypertrophic and upper hypertrophic zones) can be isolated. This allows the study of cells at two different stages of maturation. Considerable insight regarding the regulation of these cells has been gained by using this model. Particularly apparent is the differential responsiveness of these cells to the vitamin D_3 metabolites. Studies have shown that the response of rat costochondral chondrocytes to vitamin D_3 metabolites is a function of their state of differentiation. In general, resting zone chondrocytes respond primarily to 24R,25$(OH)_2D_3$, whereas growth zone chondrocytes respond primarily to 1α,25$(OH)_2D_3$ (Boyan et al., 1992). Maturation-dependent effects are seen by differences in extracellular protein synthesis (Schwartz et al., 1989), matrix vesicle and plasma membrane enzyme activities (Dean et al., 1996; Boyan et al., 1994; Dean et al., 1992; Schwartz and Boyan, 1988a; Schwartz et al., 1988b), cell proliferation (Schwartz et al., 1989), arachidonic acid turnover (Swain et al., 1992; Schwartz et al., 1990), prostaglandin production (Schwartz et al., 1992a), Ca^{++} flux (Langston et al., 1990), vitamin D_3 metabolite production (Schwartz et al., 1992b), and protein kinase C activity (Sylvia et al., 1993). 1α,25$(OH)_2D_3$ treatment of growth zone chondrocyte cultures increases alkaline phosphatase (Schwartz and Boyan, 1988a) and phospholipase A_2 specific activities (Schwartz et al., 1988b), as well as prostaglandin E_2 production (Schwartz et al., 1992a). By contrast, 24R,25$(OH)_2D_3$ treatment of resting zone chondrocyte cultures increases alkaline phosphatase specific activity (Schwartz and Boyan, 1988a), decreases phospholipase A_2 specific activity (Schwartz et al., 1988b), and decreases prostaglandin E_2 production (Schwartz et al., 1992a). In addition, 24R,25$(OH)_2D_3$ regulates the differentiation and maturation of resting zone chondrocytes into growth zone chondrocytes (Schwartz et al., 1995). 24R,25$(OH)_2D_3$ causes resting zone

cells to mature along the endochondral developmental pathway, where they become more responsive to $1\alpha,25(OH)_2D_3$ and lose responsiveness to $24R,25(OH)_2D_3$, a characteristic of more mature growth zone chondrocytes.

$1\alpha,25(OH)_2D_3$ elicits its effects on growth zone cells through different signal transduction pathways than those used by $24R,25(OH)_2D_3$ (Boyan et al., 2001c). Although both metabolites stimulate protein kinase C (PKC) specific activity, they do so only in their target cells (Sylvia et al., 1993). $1\alpha,25(OH)_2D_3$ increases PKC-alpha (PKCα) activity in growth zone cells, but has no effect on PKC in resting zone cells. $24R,25(OH)_2D_3$ increases PKCα activity in resting zone cells, but has no effect on PKC in growth zone cells. The vitamin D metabolites exert their stimulatory effects via different mechanisms. $1\alpha,25(OH)_2D_3$ causes a rapid increase in phospholipase C (PLC) activity via a mechanism involving phospholipase A_2 (PLA_2) activation (Schwartz et al., 1988b). $24R,25(OH)_2D_3$ causes a rapid decrease in PLA_2 activity and activates phospholipase D (PLD) (Boyan et al., 2001c), leading to diacylglycerol (DAG) production (Boyan et al., 2001c). The effects of $1\alpha,25(OH)_2D_3$ are non-genomic. DAG produced via PLC action binds PKC and causes its translocation to the plasma membrane. In contrast, $24R,25(OH)_2D_3$ -dependent increases include both rapid activation of existing PKC and a genomic mechanism. As a result, peak increases in PKC due to $1\alpha,25(OH)_2D_3$ occur within 9 minutes, but the $24R,25(OH)_2D_3$ effect is maximal at 90 minutes.

PKC signaling cascades can lead to genomic responses through a variety of pathways, including mitogen-activated protein kinase. In other cell types, $1\alpha,25(OH)_2D_3$ was shown to activate MAP kinase, and MAP kinase could also be activated by analogues of $1,25(OH)_2D_3$ that exhibit very low binding affinity for the nuclear vitamin D receptor (de Boland and

Norman, 1988). PKC may be necessary but not sufficient for MAP kinase response to $1\alpha,25(OH)_2D_3$. Stimulation of MAP kinase and, further upstream, of Raf kinase by $1\alpha,25(OH)_2D_3$ via a PKC-dependent pathway have been recently reported in other cell types (Beno et al., 1995; Lissoos et al., 1993). The mechanism by which PKC activates the MAP kinase cascade is unclear, although it has been suggested that PKC directly phosphorylates and activates Raf in a p21 ras-independent manner (Kolch et al., 1993). PKC is used by many receptor types to regulate the MAP kinase pathway, alone or with other mechanisms, and may act at several steps in the cascade (Cobb and Goldsmith, 1995).

Mitogen-activated proteins are activated in response to a variety of extracellular stimuli and shown to be involved in cell growth, transformation, differentiation, and apoptosis (Takahashi and Berk, 1998). More than a dozen mammalian MAP kinase family members have been discovered. MAP kinases are found within protein kinase cascades, downstream from PKC, and they are activated by dual phosphorylation on tyrosine and threonine residues. Cobb (1999) has described these cascades in the following way:

> "Each cascade consists of no fewer than three enzymes that are activated in series: the MAP kinase or ERK (extracellular signal-regulated protein kinase) is activated by a MAP/ERK kinase or MEK which is activated by a MEK kinase or MEKK. This kind of three-kinase regulatory cascade not only conveys information to target effectors, but also coordinates incoming information from parallel signaling pathways, allows for signal amplification, generates a threshold and a sigmoid activation profile, is subject to multiple inactivation mechanisms, and accommodates a variety of mechanisms for control of subcellular localization."

These enzymes make up a MAP kinase module. MAP kinase modules integrate signals coming from membrane receptor activation. When they are activated they can translocate into the nucleus (Chen et al., 1992; Lenormand et al., 1993; Gonzalez et al., 1993; Cheng et al., 1996), where they phosphorylate and activate nuclear targets, such as

transcription factors (Blenis, 1993). Thus they relay extracellular signals into a genomic response (Brunet and Pouysségur, 1997). As a result, MAP kinases may have roles in regulation of protein phosphorylation in the nucleus as well as in the cytoplasm (Pelech and Sanghera, 1992; Roberts, 1992). Cobb (1999) noted that:

> MAP kinase substrates may be regulated by one or several MAP kinase pathways. Two or more MAP kinases may stimulate the same downstream effectors. Because substrates may be shared, signals from individual cascades are integrated not only during early signal transduction events and within the kinase cascades, but also at the level of substrate phosphorylation. The implication of this is that each substrate is a sensor of the activation state of all of the signaling pathways that impinge on it. This integration phenomenon is now known to be common for protein kinase substrates.

Since both threonine and tyrosine must be phosphorylated for high MAP kinase activity, removing phosphate from either site will inactivate it. MAP kinase phosphatases (MKPs) are a group of dual specificity phosphatases related to the tyrosine phosphatase gene family (Guan and Dixon, 1993; Zheng and Guan, 1993; Sun et al., 1993; Alessi et al., 1993; Ward et al., 1994; Martell et al., 1995; Chu et al., 1996). Cobb (1999) stated that

> These phosphatases may act in a stimulus-dependent manner, may themselves be temporarily regulated, may control only a subpopulation of MAP kinase activity due to compartmentalization or co-localization, and almost certainly act not singly, but in groups to provide appropriate inactivation of MAP kinases.

These enzymes can dephosphorylate both phosphoresidues of MAP kinase family members (English, et al, 1999).

The current study is based on the hypothesis that MAP kinase activity in growth zone and resting zone chondrocytes mediates at least some of the effects of $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ in a cell maturation-dependent and vitamin D_3 metabolite-specific manner, and that it is regulated via PKC.

Purpose of Investigation: To prove or disprove the study's hypothesis, the following specific aims were pursued.

Specific Aim 1: To determine if MAP kinase is expressed in growth zone and resting zone chondrocytes. Both types of chondrocytes were cultured to fourth passage and harvested for determination of MAP kinase protein production by Western blot, Northern blot, and enzyme activity.

Specific Aim 2: To determine if 24R,25$(OH)_2D_3$ regulates MAP kinase activity in resting zone chondrocytes, and if 1α,25$(OH)_2D_3$ regulates MAP kinase activity in growth zone chondrocytes. Resting zone chondrocytes were treated for 9, 90, and 120 minutes with 10^{-9}, 10^{-8}, and 10^{-7} M 24R,25$(OH)_2D_3$ and growth zone chondrocytes were treated with 10^{-10}, 10^{-9}, and 10^{-8} M 1α,25$(OH)_2D_3$ for the same time intervals. At harvest, MAP kinase activities were determined.

Specific Aim 3: To determine if MAP kinase mediates the effect of 24R,25$(OH)_2D_3$ in resting zone chondrocytes and 1α,25$(OH)_2D_3$ in growth zone chondrocytes. Resting zone chondrocytes were treated with 24R,25$(OH)_2D_3$ at the optimal dose and time found in Specific Aim 2 in the presence and absence of an inhibitor of the MAP kinase pathway. At harvest, cell number and [^{3}H]-thymidine incorporation were measured to assess changes in cell proliferation; alkaline phosphatase specific activity was measured to assess changes in differentiation; [^{35}S]-SO_4 incorporation was measured to assess changes in matrix synthesis; and TGF-β1 and PGE_2 were measured to assess changes in local factor levels. Growth zone chondrocytes were treated with 1α,25$(OH)_2D_3$ at the optimal dose and time and analyzed.

Specific Aim 4: To determine which signaling pathways are used by the vitamin D_3 metabolites to enter into the MAP kinase pathway in resting zone and growth zone

chondrocytes. Inhibitors specific for protein kinase A, protein kinase C, phosphatidylinositol-specific phospholipase C, phospholipase D, and indomethacin were employed. The role of the membrane receptor for $1\alpha,25(OH)_2D_3$ was assessed using a blocking antibody specific for this receptor.

MATERIALS AND METHODS

Reagents

1α,25$(OH)_2D_3$ and 24R,25$(OH)_2D_3$ were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). 1β,25$(OH)_2D_3$ and 24S,25$(OH)_2D_3$ were generous gifts of Dr. Anthony W. Norman (University of California Riverside, Riverside, CA). Ab99, a rabbit polyclonal antibody generated to the N-terminal amino acid sequence of the [^{3}H]-1α,25$(OH)_2D_3$ binding protein in the basal lateral membranes of chick intestinal epithelium (Nemere et al., 2000) was a gift from Dr. Ilka Nemere (Utah State University, Logan, UT). The following chemicals were purchased from Calbiochem (San Diego, CA): PD98059 (MEK inhibitor) (Wheeler-Jones, 1996), chelerythrine and H-7 (PKC inhibitors) (Herbert et al., 1990; Kawamoto and Hidaka, 1984), H-89 (PKA inhibitor) (Chijawa et al., 1990), U73122 (phosphatidylinositol-specific phospholipase C inhibitor) (Bleasdale et al., 1989), wortmannin (PLD inhibitor) (Carrasco-Marin et al., 1994), actinomycin D (transcription inhibitor), and cycloheximide (translation inhibitor). Indomethacin (cyclooxygenase inhibitor) was purchased from Sigma Chemical Co. (St. Louis, MO). The Biotrack p42/p44 MAP Kinase assay kit was purchased from Amersham Life Science (Piscataway, NJ). Dulbecco's modified Eagle medium (DMEM) was obtained from GIBCO-BRL (Gaithersburg, MD) and fetal bovine serum (FBS) was purchased from Atlanta Biological (Norcross, GA). The protein content of each sample was determined using the bicinchoninic acid (BCA) protein assay reagent (Smith et al., 1985) obtained from Pierce Chemical Co. (Rockford, IL). [^{32}P]-ATP, [^{3}H]-thymidine and [^{35}S]-sulfate were obtained from NEN-DuPont (Boston, MA).

Chondrocyte Cultures

The culture system used in this study has been described in detail previously (Boyan et al., 1988; Schwartz et al., 1988b; Schwartz et al., 1988c). Chondrocytes were isolated from the resting zone (reserve zone) and growth zone (prehypertrophic/upper hypertrophic cell zones) of the costochondral junction of 125 g male Sprague-Dawley rats and cultured in DMEM containing 10% FBS and 50 μg/ml vitamin C in an atmosphere of 5% CO_2 and 100% humidity at 37°C. Fourth passage cells were used for all experiments, since prior studies have shown retention of differential phenotype at this number of passages (Boyan et al., 2001; Boyan et al., 1997). At confluence, media were removed and replaced with experimental media containing $1\alpha,25(OH)_2D_3$ or $24R,25(OH)_2D_3$ at the concentrations indicated below.

MAP Kinase mRNA Levels

RT-PCR

To determine whether chondrocytes express genes for ERK-1 and ERK-2, RNA preparations from resting zone and growth zone cell cultures were screened by RT-PCR. Total RNA was isolated with TRIzol reagent (Gibco-BRL). For sequence determination, total RNA from resting zone and growth zone cells were reverse transcribed with the First-strand cDNA synthesis kit (Pharmacia) and sequenced by the Center for Advanced DNA Technologies in the Department of Microbiology at The University of Texas Health Science Center at San Antonio. For rat brain, kidney, and liver RNA samples, 1 μg of total RNA (purchased from Ambion, Austin, TX)) was used in reverse transcriptase reactions. Rat primer sequences were generated from rat sequences (available in GenBank) and based on

primer sequences shown to amplify rat ERK cDNAs. The sets of ERK primers were: ERK-1 sense, 5'-GAT TGC TGA CCC TGA GCA C-3', and ERK-1 antisense, 5'-GGG GGC CTC TGG TGC C-3'; ERK-2 sense, 5'-GCC CGG AGA TGG TCC GC-3', and ERK-2 antisense, 5'-ATG GTC TGG ATC TGC AAC A-3'. The expected product sizes were 570 base pairs (ERK-1) and 506 base pairs (ERK-2). Rat brain RNA served as positive control template for ERK-1 and ERK-2. Sequences were confirmed by direct comparison with published rat ERK-1 and ERK-2 sequences available in GenBank [ERK-1, accession number M61177 (Maisonpierre et al., 1991)]; ERK-2, accession number M64300 (Boulton et al., 1991)].

Northern Blot Analysis

To quantitate the effects of $1\alpha,25(OH)_2D_3$ on mRNA levels for ERK-1 and ERK-2, we performed Northern blot analysis. Total RNA for untreated and $1\alpha,25(OH)_2D_3$-treated growth zone chondrocytes, as well as from untreated and $24R,25(OH)_2D_3$-treated resting zone cells, was isolated with TRIzol. Total RNA was quantitated spectrophotometrically, separated on a 1% denaturing agarose gel, and transferred to a positively charged nylon membrane (Ambion) with the Turboblotter System (Schleicher and Schuell, Keen, NH). Northern blots were hybridized with ERK-1, ERK-2 and GAPDH strippable [^{32}P]-labeled anticoding RNA probes using the NorthernMax Kit (Ambion). ERK-1 and ERK-2 DNA templates were synthesized from sequenced RT-PCR products amplified with modified antisense primers with the T7 promoter sequence, 5'-TAA TAG GAC TCA CTA TAG GGA GG-3', attached to the 5' end of the antisense primers. Anticoding RNA probes were synthesized with the Strip-EZ T7 Kit (Ambion). Northern blots were analyzed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Requirement for Gene Expression and Protein Synthesis

To determine if new gene expression or protein synthesis is required for $1\alpha,25(OH)_2D_3$- and $24R,25(OH)_2D_3$-dependent stimulation of MAP kinase activity, growth zone and resting zone chondrocytes were treated with 1, 10, or 100 μM actinomycin D to block transcription, or with 1, 10, or 100 μM cycloheximide to block translation, as described previously (Sylvia et al., 1993; Obrig et al., 1971).

Regulation of MAP Kinase by $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$

MAP Kinase Specific Activity

Mitogen activated protein kinase (MAP kinase) specific activity was determined using the Biotrack p42/p44 MAP Kinase assay kit following the manufacturer's directions. To determine if the regulation of MAP kinase by $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ was metabolite-specific and cell-specific, cultures were treated with the different vitamin D_3 metabolites. For growth zone cells, cultures in 24-well plates were treated for various time periods with 0.5 ml of vehicle control (0.02% ethanol in DMEM + 10% FBS) or experimental DMEM + 10% FBS plus 10^{-8}M $1\alpha,25(OH)_2D_3$, or for 9 minutes with 10^{-10}, 10^{-9} or 10^{-8} M $1\alpha,25(OH)_2D_3$, $1\beta,25(OH)_2D_3$, or $24R,25(OH)_2D_3$. For resting zone cells, cultures in 24-well plates were treated for various periods of time with 0.5 ml of vehicle control (0.02% ethanol in DMEM + 10% FBS) or experimental DMEM + 10% FBS plus 10^{-7}M $24R,25(OH)_2D_3$, or for 90 minutes with 10^{-9}, 10^{-8} or 10^{-7} M $24R,25(OH)_2D_3$, $24S,25(OH)_2D_3$, or $1\alpha,25(OH)_2D_3$. After the appropriate incubation period, cell layers were washed with phosphate buffered saline (PBS) and lysed in solubilization buffer (50 mM Tris-HCl, pH 7.5,

150 mM NaCl, 5 mM EDTA, and 1% NP-40) for 30 minutes on ice. The cell layer lysates were assayed for protein content (Smith et al., 1985) and MAP kinase activity. Fifteen microliters of each experimental sample was mixed with ten microliters of substrate buffer and five microliters of Mg/ATP reagent, both provided in the kit. Immediately prior to use, [^{32}P]-ATP was added to the Mg/ATP reagent to a final concentration of 200 μCi/ml. Following a 30-minute incubation at 30°C, the reaction was stopped by addition of 10 μl of stop reagent, the samples were centrifuged for 15 seconds at 14,000 rpm, and 30 μl spotted onto phosphocellulose discs. The discs were washed twice with 75 mM orthophosphoric acid and once with distilled water, and counted in a liquid scintillation counter.

Western Blot Analysis

To determine if 1α,25(OH)$_2$D$_3$ or 24R,25(OH)$_2$D$_3$ regulated the levels of ERK1/2 protein or if MAP kinase was activated by tyrosine phosphorylation (Cobb, 1999), cell culture lysates were examined by Western blot using specific antibodies to non-phosphorylated and phosphorylated p42/p44. Cell culture lysates were prepared from confluent, fourth passage growth zone cell cultures that had been treated for 1, 9, or 90 minutes with 10^{-8} M 1α,25(OH)$_2$D$_3$, or from resting zone cell cultures treated for 1, 9, 90 or 270 minutes with 10^{-7} M 24R,25(OH)$_2$D$_3$, and were resolved on 10% SDS-polyacrylamide gels. Similar gels were run using cell culture lysates prepared from growth zone cell cultures treated for 9 minutes with 10^{-10} M, 10^{-9} M or 10^{-8} M 1α,25(OH)$_2$D$_3$, or from resting zone cell cultures treated for 90 minutes with 10^{-9} M, 10^{-8} M or 10^{-7} M 24R,25(OH)$_2$D$_3$. Blots of the gels were probed with 1:5000 dilutions of rabbit polyclonal antibodies to phosphorylated p42/p44 or non-phosphorylated p42/p44 (Promega Corp., Madison, WI), followed by 1:5000

dilutions of alkaline phosphatase-conjugated anti-rabbit IgG1 (Santa Cruz Biotechnology, Santa Cruz, CA). The ERK-1 (44 kD) and ERK-2 (42 kD) bands were visualized using the NBT/BCIP reagent (Sigma Corporation, St. Louis, MO).

Role of MAP Kinase in Mediating the Physiologic Response to Vitamin D_3 Metabolites

[^{3}H]-Thymidine Incorporation

Vitamin D_3 metabolites inhibit [^{3}H]-thymidine incorporation by resting zone and growth zone chondrocytes (Boyan et al., 1992; Schwartz et al., 1989). To determine if this response is mediated by MAP kinase, DNA synthesis was estimated by measuring [^{3}H]-thymidine incorporation into trichloroacetic acid (TCA) insoluble cell precipitates as described previously (Schwartz et al., 1989). Quiescence was induced by incubating confluent cultures for 48 hours in DMEM containing 1% FBS. The medium was then replaced with DMEM containing 1% FBS alone (control), 10^{-10} to 10^{-8} M 1α,25$(OH)_2D_3$ for growth zone cells, or 10^{-9} to 10^{-7} M 24R,25$(OH)_2D_3$ for resting zone cells, in the presence or absence of 1, 10, or 100 μM PD98059 for 24 hours. PD98059 prevents activation of MAP kinase by inhibiting the action of MEK, which is the enzyme that phosphorylates ERK1/2 (Wheeler-Jones et al., 1996). Two hours prior to harvest, [^{3}H]-thymidine was added.

Proteoglycan Sulfation

1α,25$(OH)_2D_3$ causes an increase in [^{35}S]-sulfate incorporation in growth zone chondrocyte cultures, whereas 24R,25$(OH)_2D_3$ causes an increase in [^{35}S]-sulfate incorporation in cultures of resting zone cells (Schwartz et al., 1995). To determine if this is mediated by MAP kinase, proteoglycan synthesis was assessed by measuring [^{35}S]-sulfate incorporation by confluent cultures as described previously (Nasatsky et al., 1994; O'Keefe

et al., 1988). At confluence, fresh medium containing vehicle alone, 10^{-10} to 10^{-8} M $1\alpha,25(OH)_2D_3$ for growth zone cell cultures or 10^{-9} to 10^{-7} M $24R,25(OH)_2D_3$ for resting zone cell cultures in the presence or absence of 1, 10 or 100 μM PD98059 (MEK inhibitor) for 24 hours. Four hours prior to harvest, 50 μl DMEM containing 18 μCi/ml [^{35}S]-sulfate and 0.814 mM carrier sulfate were added to each culture. At harvest, the conditioned media were removed, the cell layers (cells and matrix) collected, and the amount of [^{35}S]-sulfate incorporated determined as a function of cell layer protein.

Alkaline Phosphatase Specific Activity

$1\alpha,25(OH)_2D_3$ stimulates alkaline phosphatase specific activity in growth zone chondrocyte cultures, whereas $24R,25(OH)_2D_3$ increases this enzyme activity in resting zone cell cultures (Schwartz et al., 1988b). To determine if this effect is mediated by MAP kinase, confluent cultures were treated with medium containing vehicle alone, 10^{-10} to 10^{-8} M $1\alpha,25(OH)_2D_3$ for growth zone cells or 10^{-9} to 10^{-7} M $24R,25(OH)_2D_3$ for resting zone cells in the presence or absence of 1, 10, or 100 μM PD98059 (MEK inhibitor) for 24 hours. Alkaline phosphatase [orthophosphoric monoester phosphohydrolase, alkaline (EC 3.1.3.1)] specific activity was measured in cell layer lysates as a function of release of *para*-nitrophenol from *para*-nitrophenylphosphate at pH 10.2, as described previously (Schwartz et al., 1988; Hale et al., 1986; Bretaudiere and Spillman, 1984).

Protein Kinase C and Protein Kinase A

$1\alpha,25(OH)_2D_3$ mediates its effects on growth zone cells via the PKC and PKA signaling pathways (Boyan et al., 1999). These pathways also mediate the effects of

24R,25$(OH)_2D_3$ on resting zone cells (Boyan et al., 2001). To determine if activation of MAP kinase involved either one or both mechanisms, specific inhibitors of each pathway were used. Two different inhibitors were used to inhibit PKC, chelerythrine (0.1, 1 or 10 μM) (Herbert et al., 1990) and H-7 (0.1 or 1μM) (Kawamoto and Hidaka, 1984). Growth zone chondrocyte cultures were incubated for 9 minutes in control media or media containing 10^{-8} M 1α,25$(OH)_2D_3$ ± chelerythrine or H-7, and resting zone cultures were incubated for 90 minutes in control media or media containing 10^{-7} M 24R,25$(OH)_2D_3$ ± chelerythrine or H-7 and MAP kinase activity determined. The involvement of PKA was assessed in a similar manner using the PKA inhibitor H-89 (0.1 or 1 μM) (Chijiwa et al., 1990).

Phospholipase C

Phospholipase C (PLC) mediates the effects of 1α,25$(OH)_2D_3$ on PKC in growth zone cell cultures (Sylvia et al., 1998), but does not mediate the effects of 24R,25$(OH)_2D_3$ on resting zone cells (Schwartz et al., 2001). Its involvement in the effect of vitamin D_3 metabolites on MAP kinase was assessed by using U73122, an inhibitor of PI-PLC (Bleasdale et al., 1989), at 0.1, 1.0, or 10 μM. Growth zone chondrocytes were incubated for 9 minutes in control media or media containing 10^{-8} M 1α,25$(OH)_2D_3$ ± U73122, and resting zone cell cultures were incubated for 90 minutes in control media or media containing 10^{-7} M 24R,25$(OH)_2D_3$ ± U73122.

Phospholipase D

PLD mediates the membrane effects of 24R,25$(OH)_2D_3$ on PKC activity of resting zone cells, but not growth zone cells (Schwartz et al., 2001). Its involvement in the effects of

vitamin D_3 metabolites on MAP kinase activity was assessed using wortmannin, an inhibitor of PLD (Carrasco-Marin et al., 1994; Mollindo et al., 1994). Growth zone cell cultures were incubated for 9 minutes in control media or media containing 10^{-8} M $1\alpha,25(OH)_2D_3 \pm 0.1$, 1.0, or 10 μM wortmannin. Resting zone cells were incubated for 90 minutes in control media or media containing 10^{-7} M $24R,25(OH)_2D_3 \pm$ 0.1, 1.0, or 10 μM wortmannin.

Cyclooxygenases

Inhibition of cyclooxygenase-1 blocks the effects of $1\alpha,25(OH)_2D_3$ on PKC in growth zone cells and augments the effects of $24R,25(OH)_2D_3$ in resting zone cells (Sylvia et al., 2001; Schwartz et al., 2000). Involvement of prostaglandins in the regulation of MAP kinase by these metabolites was assessed by using indomethacin, a general inhibitor of cyclooxygenases. Growth zone cells were incubated for 9 minutes in control media or media containing 10^{-8} M $1\alpha,25(OH)_2D_3 \pm 10^{-8}$ M or 10^{-7} M indomethacin. Resting zone cells were incubated for 90 minutes in control media or media containing 10^{-7} M $24R,25(OH)_2D_3 \pm 10^{-8}$ M or 10^{-7} M indomethacin.

Statistical Management of the Data

For each experiment, each data point represents the mean ± standard error of the mean (SEM) of six independent cultures. Observations are verified by a minimum of two experimental replications. Differences between groups will be determined by ANOVA, and significance between groups determined by use of Bonferroni's modifications of Student's t-test using $P<0.05$ confidence limits.

RESULTS

Resting zone and growth zone chondrocytes express genes for ERK-1 and ERK-2 as shown by screening RNA preparations by RT-PCR (Figure 1), and the amounts are quantitatively similar. The effect of the vitamin D_3 metabolites on MAP kinase activity was not due to a change in gene expression in either cell type (Figure 2). No change in p44 or p42 mRNA levels were noted in Northern blots of RNA isolated from growth zone cells treated with $1\alpha,25(OH)_2D_3$ at any of the time points (9 and 90 minutes and 24 hours) examined. Similarly, $24R,25(OH)_2D_3$ did not affect mRNA levels in resting zone cells. Protein synthesis was also not responsible for the change in enzyme activity. Neither cycloheximide nor actinomycin D altered MAP kinase activity in control cultures or in cultures treated with $1\alpha,25(OH)_2D_3$ or $24R,25(OH)_2D_3$ (data not shown). Moreover, there were no changes in p42 or p44 protein levels in Western blots of the cell lysates. In contrast, phosphorylation of ERK-1 and ERK-2 was regulated by the vitamin D_3 metabolites in a time and dose-dependent manner (Figure 3). Phosphorylated p42 and p44 were evident in growth zone cells treated with 10^{-8} M $1\alpha,25(OH)_2D_3$ as early as 1 minute, and maximal phosphorylation was achieved at 90 minutes. The phosphorylation was seen in cultures treated with 10^{-10} M, particularly the p42 band, and maximal phosphorylation was seen in cultures treated with 10^{-8} M $1\alpha,25(OH)_2D_3$. The effect of $24R,25(OH)_2D_3$ on the phosphorylation of MAP kinase in resting zone cells was similar. Phosphorylated p42 and p44 were evident within 1 minute and increased over time, becoming maximal at 90 minutes. By 270 minutes, there was no more evidence of phosphorylated enzyme. Phosphorylation was dose-dependent, with the greatest amount in cells cultured in the presence of 10^{-7} M $24R,25(OH)_2D_3$.

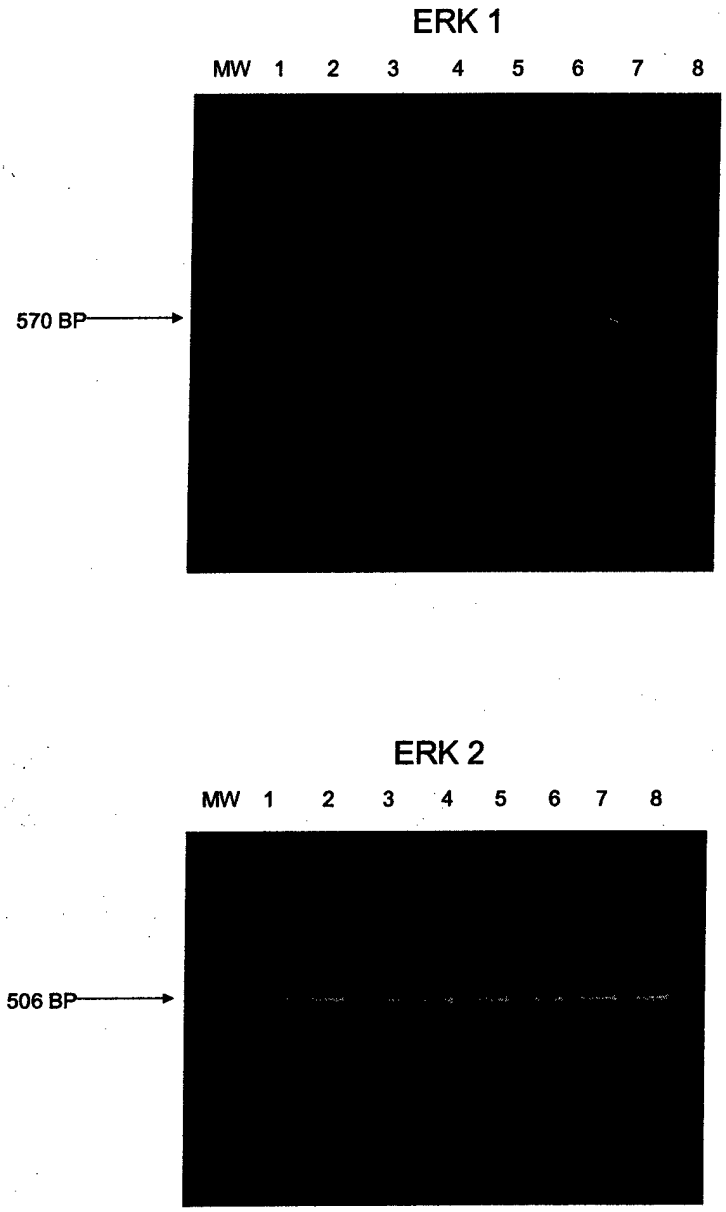


Figure 1: RT-PCR of resting zone and growth zone chondrocytes, demonstrating expression of mRNA for ERK-1 and ERK-2.

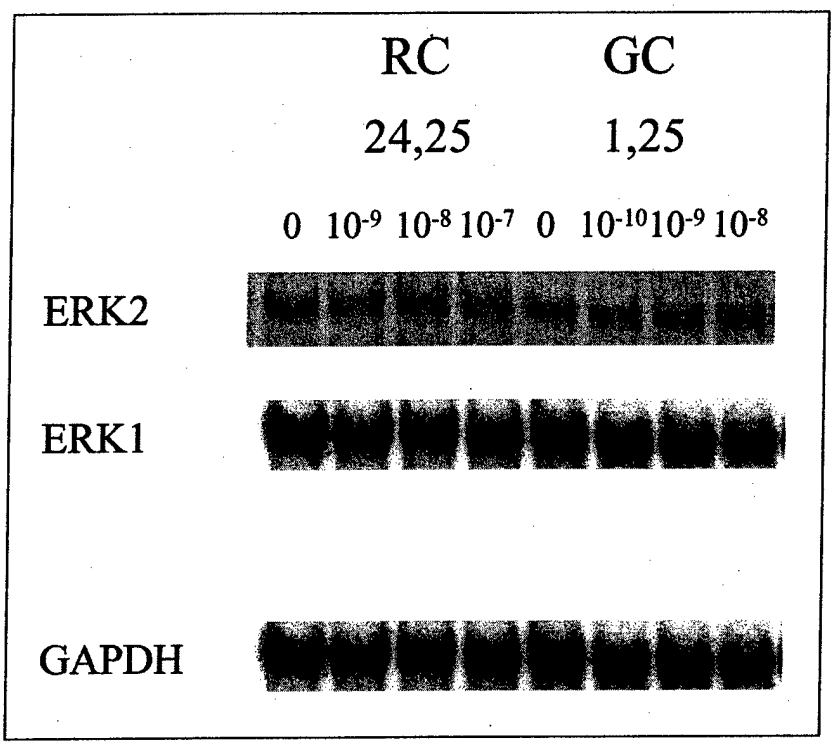


Figure 2: Effect of $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ on mRNA levels for ERK-1 and ERK-2 MAP kinase in rat costochondral growth plate chondrocytes. Confluent cultures of growth zone cells were treated for 90 minutes with 10^{-10} to 10^{-8} M $1\alpha,25(OH)_2D_3$ and confluent resting zone cells were treated with 10^{-9} to 10^{-7} M $24R,25(OH)_2D_3$ for the same length of time. RNA was prepared from the cells and analyzed by Northern blot using DNA probes as described in the Methods. The relative intensity of the bands for ERK-1 and ERK-2 were compared to the intensity of GAPDH controls run simultaneously in each time.

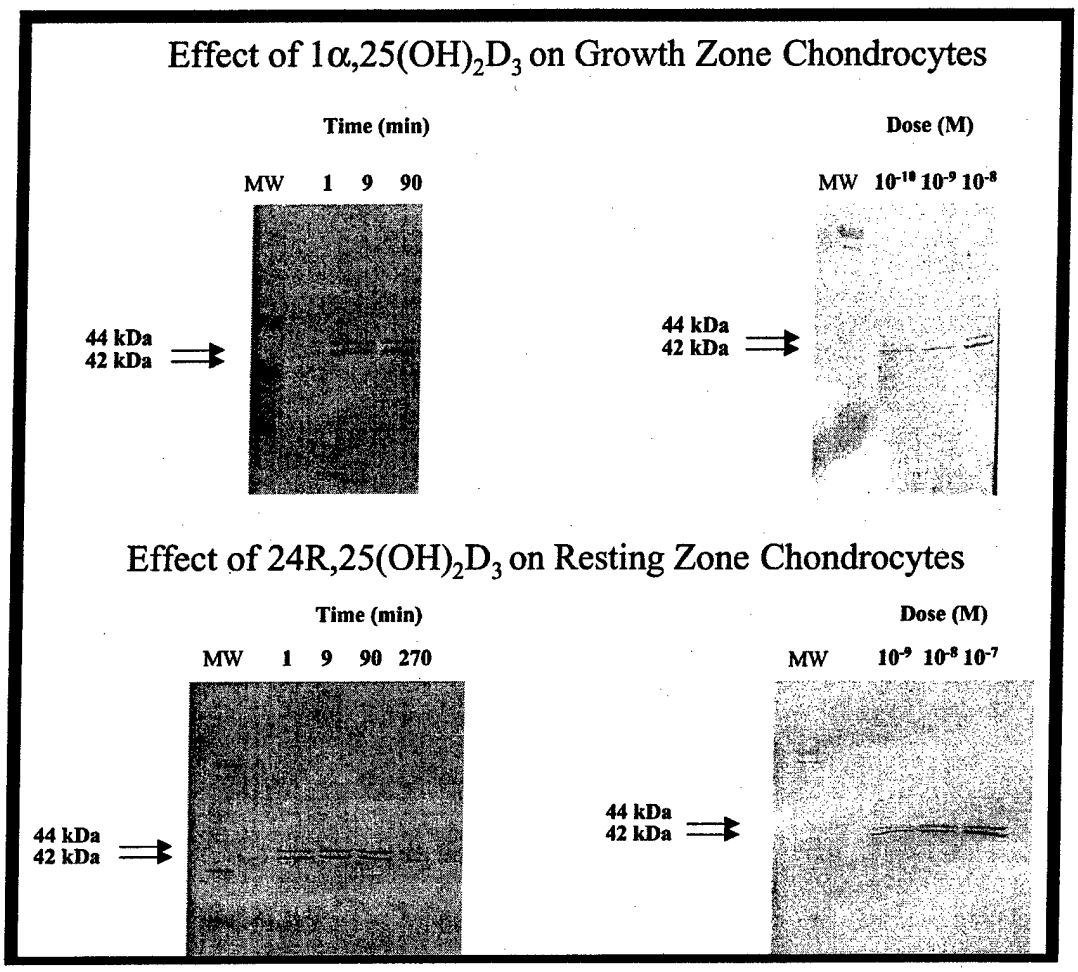


Figure 3: Western blot analysis of phosphorylated ERK-1 and ERK-2 in cell layer lysates of growth zone and resting zone chondrocyte cultures. Cell layer lysates of growth zone chondrocyte cultures that had been treated for 1, 9, or 90 minutes with 10^{-8}M 1α,25$(OH)_2D_3$ (upper left panel) or for 90 minutes with 10^{-10} to 10^{-8} M 1α,25$(OH)_2D_3$ (upper right panel) were electrophoresed on SDS polyacrylamide gels and then transferred for Western blot analysis using antibody to the phosphorylated forms of ERK-1 and ERK-2. Alternatively, cell layer lysates of resting zone chondrocyte cultures that had been treated for 1, 9, 90, or 270 minutes with 10^{-7} M 24R,25$(OH)_2D_3$ (lower left panel) or for 90 minutes with 10^{-9} to 10^{-7} M 24R,25$(OH)_2D_3$ (lower right panel) were electrophoresed, transferred, and analyzed using the same antibody.

MAP kinase specific activity in growth zone chondrocytes was regulated by $1\alpha,25(OH)_2D_3$ in dose-dependent and time-dependent manner. Figure 4 demonstrates that as the concentration of $1\alpha,25(OH)_2D_3$ increased from 10^{-10} to 10^{-8} M there was a significant increase in MAP kinase activity. Figure 5 demonstrates that 10^{-8} M $1\alpha,25(OH)_2D_3$ increased enzyme activity in 9 minutes, and peaked at 90 minutes. At 180 minutes and beyond, MAP kinase activity returned to control levels. When we examined five independent experiments and expressed the results as a treatment/control ratio, we also saw that the effects of $1\alpha,25(OH)_2D_3$ at 10^{-9} and 10^{-8} M were significantly higher than control, but 10^{-10} M was not (Figure 6).

The resting zone chondrocytes responded to $24R,25(OH)_2D_3$ in dose-dependent and time-dependent manner as well. There was a significant increase in enzyme activity at 10^{-8} and 10^{-7} M $24R,25(OH)_2D_3$ compared to control (Figure 7), and maximal at 10^{-7} M. The addition of 10^{-7} M $24R,25(OH)_2D_3$ significantly increased MAP kinase activity over control at 90, 180, and 270 minutes. The increase in activity was maximal at 90 minutes (Figure 8). Examination of five independent experiments with the results expressed as a treatment/control ratio demonstrated that the effects of $24R,25(OH)_2D_3$ at 10^{-8} and 10^{-7} M were significantly higher than control, but 10^{-9} M was not (Figure 9).

The effects of $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ are cell maturation-specific. $1\alpha,25(OH)_2D_3$ had no effect on the MAP kinase activity in the less mature resting zone cells and $24R,25(OH)_2D_3$ had no effect on MAP kinase activity in the more mature growth zone cells.

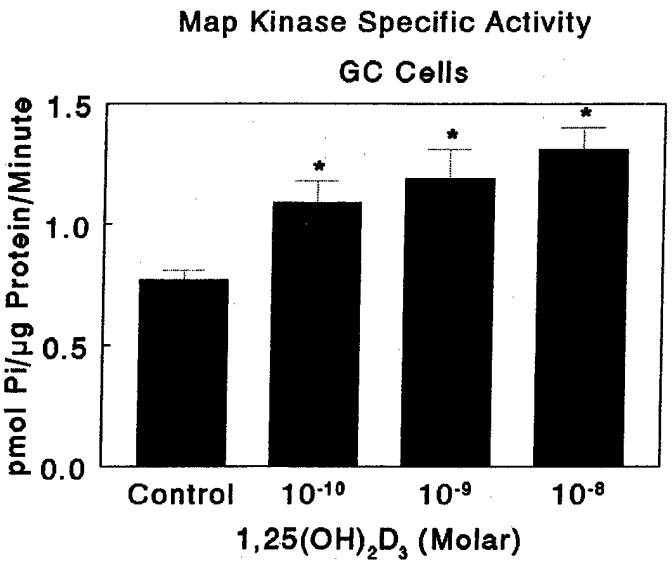


Figure 4: The effect of $1\alpha,25(OH)_2D_3$ on MAP kinase specific activity in growth zone chondrocytes (GC). Confluent fourth passage cells were treated for 9 minutes with $1\alpha,25(OH)_2D_3$ concentrations ranging from 10^{-10} to 10^{-8} M. Values are the mean ± SEM for n=6 cultures. The graph indicates that $1\alpha,25(OH)_2D_3$ had a significant dose-dependent effect on MAP kinase activity at all concentrations. $P<0.05$, vs. control vehicle.

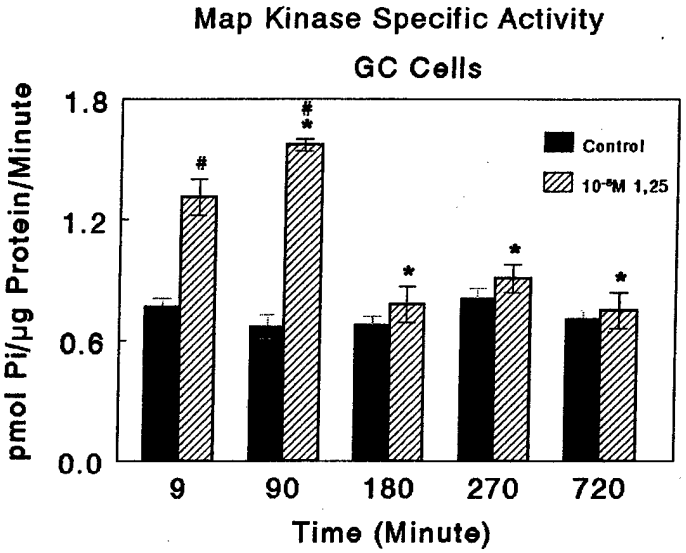


Figure 5: The effect of $1\alpha,25(OH)_2D_3$ on MAP kinase specific activity in growth zone chondrocytes over time. Confluent fourth passage cells were treated for 9 to 720 minutes with 10^{-8} M $1\alpha,25(OH)_2D_3$. Values are the mean ± SEM for n=6 cultures. The control group showed relatively stable activity from 9 to 720 minutes. The addition of $1\alpha,25(OH)_2D_3$ caused an increase in MAP kinase activity that was significant at 9 and 90 minutes (compared to control). The effect of $1\alpha,25(OH)_2D_3$ disappeared at 180, 270, and 720 minutes. *$P<0.05$, vs. 9 mins; #$P<0.05$, vs control.

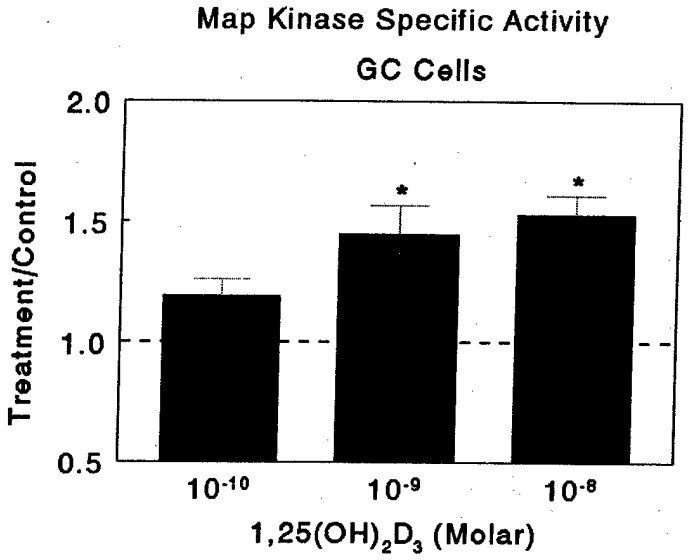


Figure 6: The effect of $1\alpha,25(OH)_2D_3$ on MAP kinase activity in five independent experiments, expressed as a treatment/control ratio. The effect of $1\alpha,25(OH)_2D_3$ at 10^{-9} and 10^{-8} M was significantly higher than control, and maximal at 10^{-8} M. *P<0.05, vs. control or T/C=1.

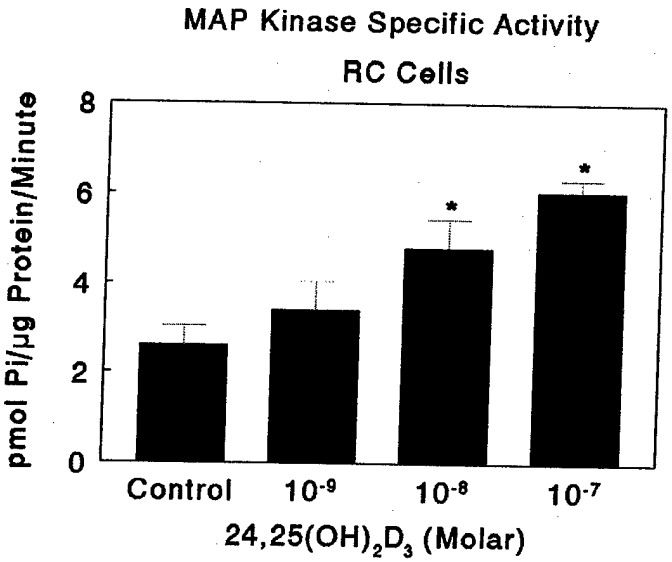


Figure 7: The effect of 24R,25$(OH)_2D_3$ on MAP kinase specific activity in resting zone chondrocytes (RC). Confluent, fourth passage cells were treated with 24R,25$(OH)_2D_3$ at concentrations ranging from 10^{-9} to 10^{-7} M. The graph indicates that 24R,25$(OH)_2D_3$ had a significant dose-dependent effect on MAP kinase activity at 10^{-8} and 10^{-7} M. Values are the mean ± SEM for n=6 cultures. $P<0.05$, vs. control vehicle.

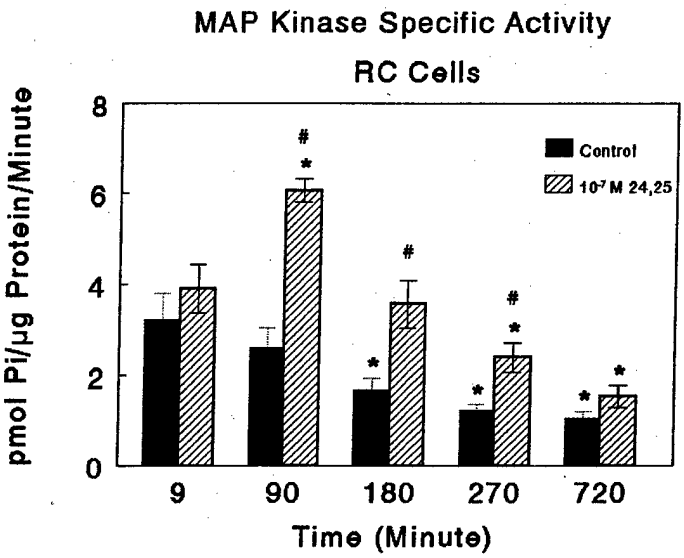


Figure 8: The effect of 10^{-7} M 24R,25$(OH)_2D_3$ on MAP kinase specific activity in resting zone chondrocytes over time. The control group showed steadily decreasing activity from 9 to 720 minutes (significant at 180, 270, and 720 minutes). The addition of 10^{-7} M 24R,25$(OH)_2D_3$ caused an increase in MAP kinase activity that was significant at 90, 180, and 270 minutes (compared to control). The decrease in effect of 24R,25$(OH)_2D_3$ became significant at 270 and 720 minutes. Values are the mean ± SEM for n=6 cultures. *P<0.05 vs. 9 mins; #P<0.05 vs. control.

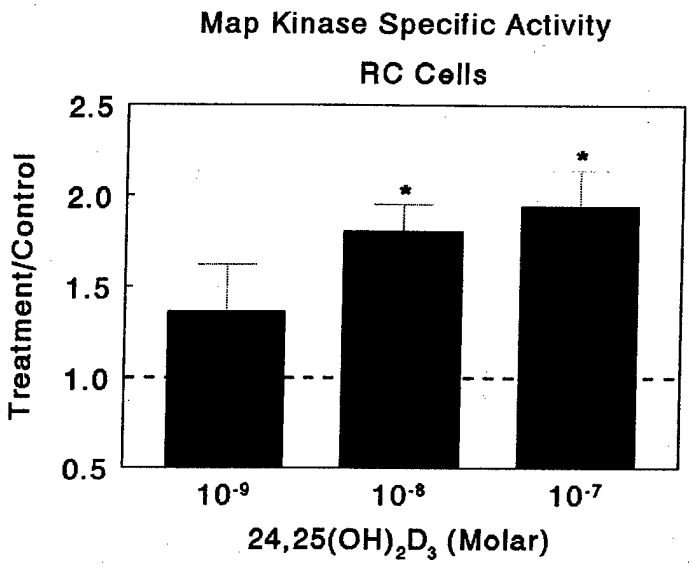


Figure 9: The effect of 24R,25$(OH)_2D_3$ on MAP kinase activity in five independent experiments, expressed as a treatment/control ratio. The effect of 24R,25$(OH)_2D_3$ at 10^{-8} and 10^{-7} M was significantly higher than control, and maximal at 10^{-7} M. *P<0.05 vs. control or T/C=1.

The MAP kinase inhibitor PD98059 reduced [^{3}H]-thymidine incorporation in both growth zone and resting zone cells. Figure 10 shows that in growth zone cells the control declined steadily with increasing inhibitor, reaching significance at 10 and 100 μM PD98059. The 1α,25(OH)$_2$D$_3$ treated cells had reduced levels of [^{3}H]-thymidine incorporation at all levels of inhibitor, and significantly less at 100 μM PD98059. In the resting zone cultures, the control cells had significantly more [^{3}H]-thymidine incorporation than the treated cells at 0, 1, and 10 μM PD98059, and significant decreases at 1, 10, and 100 μM. The treated cells showed significant decreases in [^{3}H]-thymidine incorporation at 10 and 100 μM PD98059 (Figure 11).

The effect of PD98059 in growth zone cells revealed that ERK1/2 did not play a major role in regulation of [^{35}S]-sulfate incorporation by 1α,25(OH)$_2$D$_3$. PD98059 caused a dose-dependent decrease in the controls that reached significance at 10 and 100 μM PD98059. A similar effect was seen in the 1α,25(OH)$_2$D$_3$ treated cells (Figure 12). However, the effect of 24R,25(OH)$_2$D$_3$ on [^{35}S]-sulfate incorporation by the resting zone cells was more clearly mediated by ERK1/2. In controls, the inhibitor caused a small decrease at 100 μM. In contrast, in 24R,25(OH)$_2$D$_3$ treated cells, more proteoglycan production was observed and in the presence of PD98059, a significant dose-dependent inhibition of the effect of 24R,25(OH)$_2$D$_3$ was observed at 10 and 100 μM (Figure 13).

Alkaline phosphatase activity was significantly increased by the vitamin D$_3$ metabolites in their respective cultures (compared to the controls) at all inhibitor concentrations, demonstrating that PD98059 had no effect on this enzyme (Figures 14 and 15).

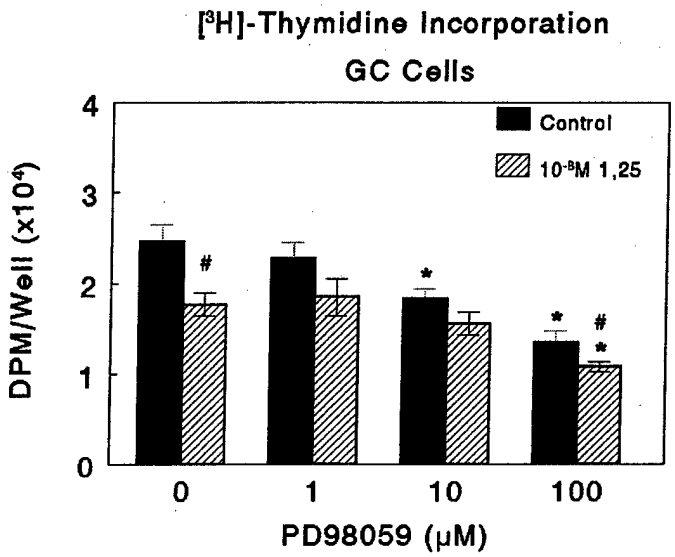


Figure 10: The effect of MAP kinase inhibitor on [^{3}H]-thymidine incorporation in growth zone chondrocytes. Cultures were treated for 24 hours with 10^{-8} M 1α,25$(OH)_2D_3$ in the presence/absence of PD98059. At harvest, [^{3}H]-thymidine incorporation was measured. The control showed a steady decline with increasing inhibitor concentration, becoming significant at 10 and 100μM PD98059. The 1α,25$(OH)_2D_3$ treated cells showed lower levels of [^{3}H]-thymidine incorporation at all inhibitor concentrations (compared to control), significantly lower at 0 and 100μM. All values are the mean ± SEM of n=6 cultures. *P<0.05 vs. no treatment with PD98059; #P<0.05 vs. no treatment with 1α,25$(OH)_2D_3$.

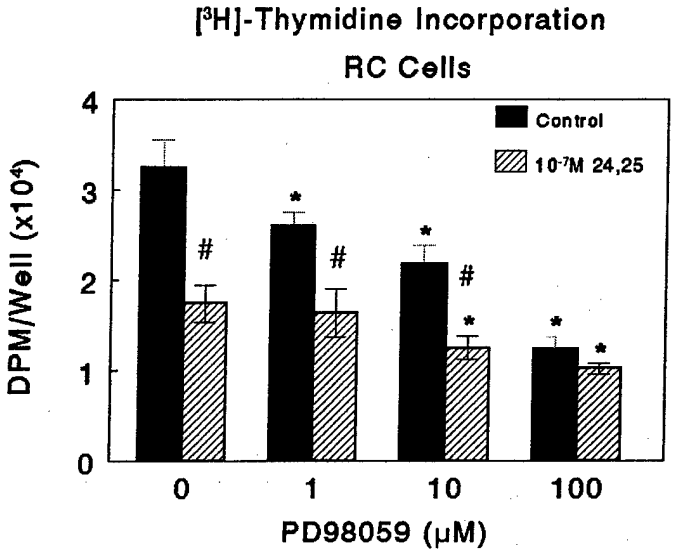


Figure 11: The effect of MAP kinase inhibitor on [^{3}H]-thymidine incorporation in resting zone chondrocytes. Cultures were treated for 24 hours with 10^{-7} M 24R,25$(OH)_2D_3$ in the presence/absence of PD98059. At harvest, [^{3}H]-thymidine incorporation was measured. The control showed a steady decline with increasing inhibitor concentration, becoming significant at 1, 10, and 100μM PD98059. The 24R,25$(OH)_2D_3$ treated cells showed lower levels of [^{3}H]-thymidine incorporation at all inhibitor concentrations (compared to control), significantly lower at 0, 1, and10μM. All values are the mean ± SEM of n=6 cultures. *P<0.05 vs. no treatment with PD98059; #P<0.05 vs. no treatment with 24R,25$(OH)_2D_3$.

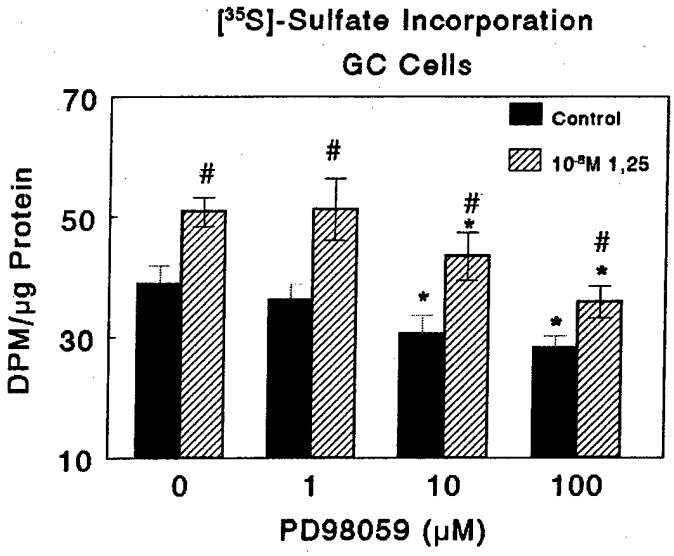


Figure 12: The effect of MAP kinase inhibitor on [^{35}S]-sulfate incorporation in growth zone chondrocytes. Cultures were treated for 24 hours with 10^{-8} M $1\alpha,25(OH)_2D_3$ in the presence/absence of PD98059. The control showed a steady decline with increasing inhibitor concentration, becoming significant at 10 and 100µM PD98059. The $1\alpha,25(OH)_2D_3$ treated cells showed significantly higher levels of proteoglycan production at all inhibitor concentrations (compared to control). The levels gradually decreased with increasing PD98059 concentration, and this decrease was significant at 100µM. Values are the mean ± SEM of n=6 cultures. *P<0.05 vs. no treatment with PD98059; #P<0.05 vs. no treatment with $1\alpha,25(OH)_2D_3$.

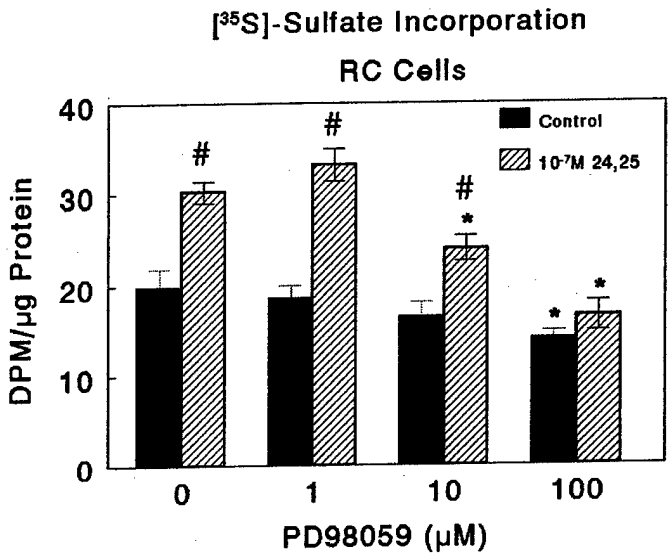


Figure 13: The effect of MAP kinase inhibitor on [^{35}S]-sulfate incorporation in resting zone chondrocytes. Cultures were treated for 24 hours with 10^{-7} M 24R,25$(OH)_2D_3$ in the presence/absence of PD98059.The control showed a steady decline with increasing inhibitor concentration, becoming significant at 100µM PD98059. The 24R,25$(OH)_2D_3$ treated cells showed significantly higher levels of proteoglycan production at 0, 1, and 10 µM inhibitor concentrations (compared to control). The levels gradually decreased with increasing PD98059 concentration, and this decrease became significant at 10 and 100µM. Values are the mean ± SEM of n=6 cultures. *P<0.05 vs. no treatment with PD98059; #P<0.05 vs. no treatment with 24R,25$(OH)_2D_3$.

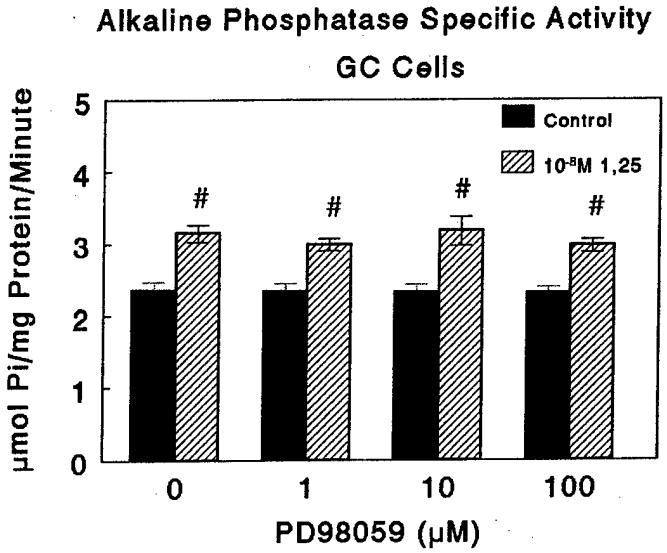


Figure 14: The effect of MAP kinase inhibitor on alkaline phosphatase specific activity in growth zone chondrocytes. The PD98059 had no effect on the control or the $1\alpha,25(OH)_2D_3$ treated cells at all inhibitor concentrations. The treated cells had a consistently and significantly higher level of activity than the controls. Values are the mean ± SEM of n=6 cultures. #$P<0.05$ vs. no treatment with $1\alpha,25(OH)_2D_3$.

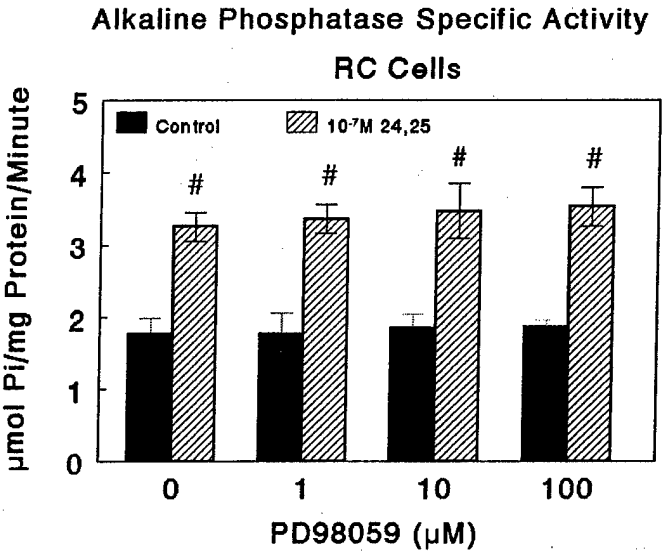


Figure 15: The effect of MAP kinase inhibitor on alkaline phosphatase specific activity in resting zone chondrocytes. The PD98059 had no effect on the control or the 24R,25$(OH)_2D_3$ treated cells at all inhibitor concentrations. The treated cells had a consistently and significantly higher level of activity than the controls. Values are the mean ± SEM of n=6 cultures. #P<0.05 vs. no treatment with 24R,25$(OH)_2D_3$.

MAP kinase activity was regulated in both growth zone and resting zone cells by a protein kinase C-dependent mechanism that did not involve protein kinase A. The PKC inhibitor chelerythrine had no effect on MAP kinase activity in growth zone control cells, but $1\alpha,25(OH)_2D_3$ treated cells demonstrated significantly higher activity at 0, 0.1, and 1 μM chelerythrine compared to controls, and a dose-dependent decrease at 1 and 10 μM (Figure 16). $24R,25(OH)_2D_3$ caused a significant increase in MAP kinase activity in resting zone cells at all concentrations of chelerythrine, as compared to controls. Here again the effect of vitamin D_3 metabolite was significantly reduced at 1 and 10 μM chelerythrine (Figure 17). Similar results were found with the PKC inhibitor H-7 (Figures 18 and 19). In addition, when the growth zone and resting zone cultures were treated with the PKA inhibitor H-89 there was no change in MAP kinase activity in the control or test cultures (Figures 20 and 21).

The effect of $1\alpha,25(OH)_2D_3$ on MAP kinase involved phospholipase C. The PLC inhibitor U73122 had no effect on growth zone cell control cultures at any concentration, but the significant increase in MAP kinase activity caused by $1\alpha,25(OH)_2D_3$ was inhibited at 1 and 10 μM U73122 (Figure 22). In the resting zone cells, the U73122 had no effect on the controls or the $24R,25(OH)_2D_3$ treated cells (Figure 23).

The phospholipase D inhibitor wortmannin had no effect on MAP kinase activity in $1\alpha,25(OH)_2D_3$ treated growth zone cells or their controls (Figure 24). However, MAP kinase activity in $24R,25(OH)_2D_3$ treated cells was significantly decreased at 1 and 10 μM wortmannin (Figure 25).

Indomethacin reduced MAP kinase activity in control cultures of growth zone cells and partially reduced the increase in enzyme activity due to $1\alpha,25(OH)_2D_3$. In contrast, indomethacin stimulated MAP kinase activity in control cultures of resting zone cells to the

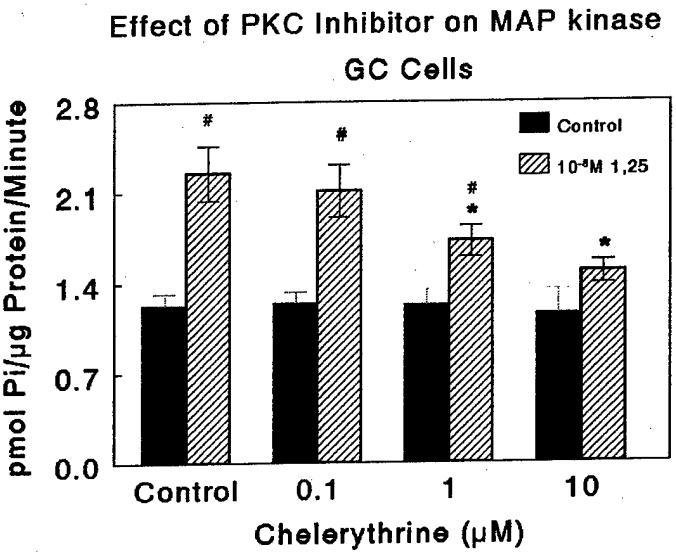


Figure 16: The effect of PKC inhibitor, chelerythrine, on MAP kinase specific activity in growth zone chondrocytes. The control showed a steady level of activity at all inhibitor concentrations. The $1\alpha,25(OH)_2D_3$ treated cells showed significantly higher levels of activity at inhibitor concentrations of 0, 0.1, and 1 µM (compared to control). The levels gradually decreased with increasing chelerythrine concentration, and this decrease was significant at 1 and10 µM. All values are the mean ± SEM for n=6 cultures. *P<0.05 vs. no treatment with chelerythrine; #P<0.05 vs. no treatment with $1\alpha,25(OH)_2D_3$.

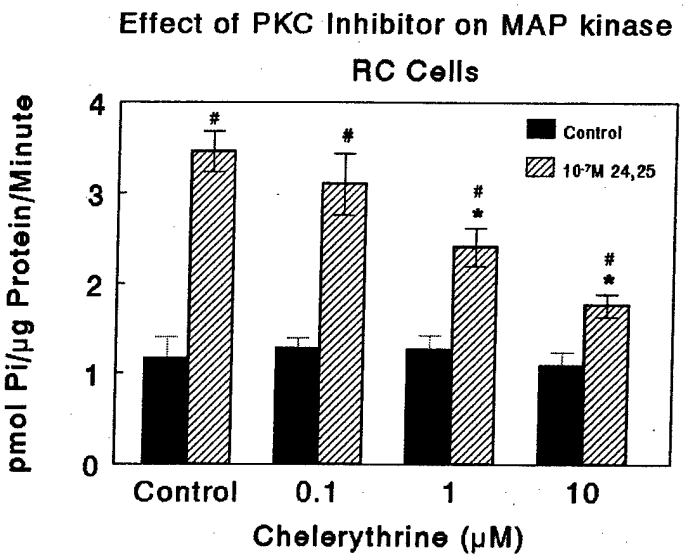


Figure 17: The effect of PKC inhibitor, chelerythrine, on MAP kinase specific activity in resting zone chondrocytes. The control showed a steady level of activity at all inhibitor concentrations. The 24R,25$(OH)_2D_3$ treated cells showed significantly higher levels of activity at all inhibitor concentrations (compared to control). The levels gradually decreased with increasing chelerythrine concentration, and this decrease was significant at 1 and10 µM. All values are the mean ± SEM for n=6 cultures. *P<0.05 vs. no treatment with chelerythrine; #P<0.05 vs. no treatment with 24R,25$(OH)_2D_3$.

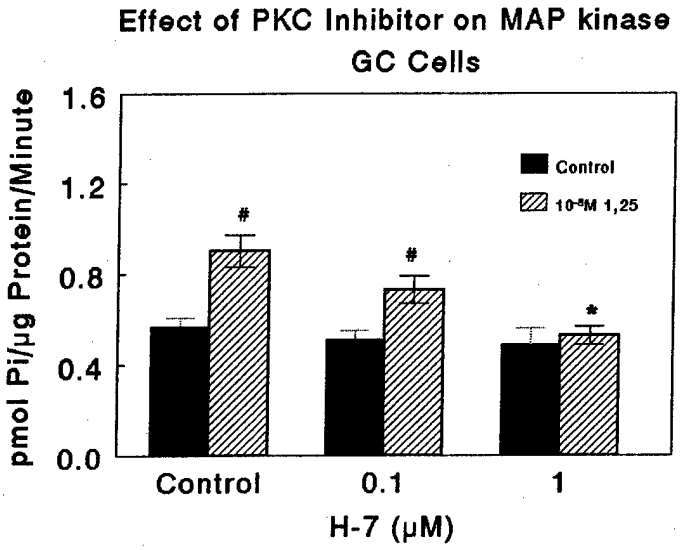


Figure 18: The effect of PKC inhibitor H-7 on MAP kinase specific activity in growth zone chondrocytes. The control showed a steady level of activity at all inhibitor concentrations. The $1\alpha,25(OH)_2D_3$ treated cells showed significantly higher levels of activity at inhibitor concentrations of 0 and 0.1 µM (compared to control). The levels gradually decreased with increasing H-7 concentration, and this decrease was significant at 1 µM. All values are the mean ± SEM for n=6 cultures. #$P<0.05$ vs. no treatment with $1\alpha,25(OH)_2D_3$.

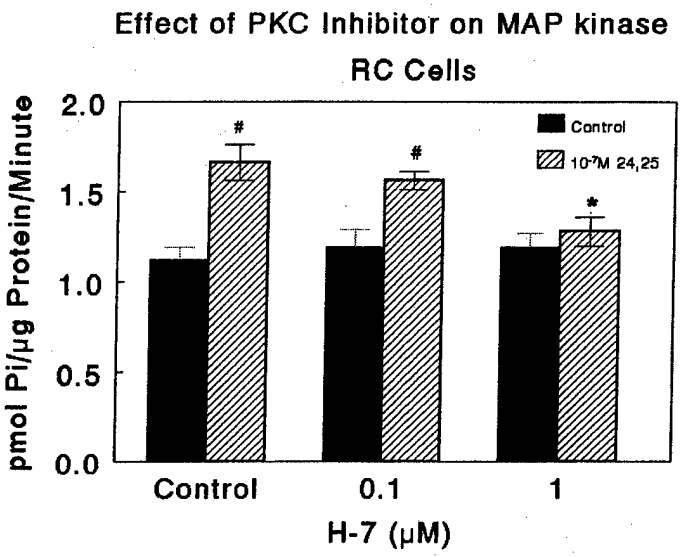


Figure 19: The effect of PKC inhibitor H-7 on MAP kinase specific activity in resting zone chondrocytes. The control showed a steady level of activity at all inhibitor concentrations. The 24R,25$(OH)_2D_3$ treated cells showed significantly higher levels of activity at 0 and 0.1 µM inhibitor concentrations (compared to control). The levels gradually decreased with increasing H-7 concentration, and this decrease was significant at 1 µM. All values are the mean ± SEM for n=6 cultures. #P<0.05 vs. no treatment with 24R,25$(OH)_2D_3$.

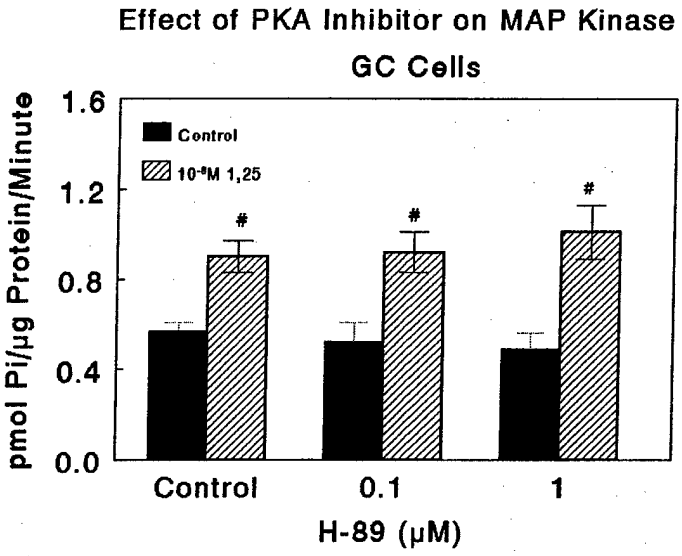


Figure 20: The effect of PKA inhibitor H-89 on MAP kinase specific activity in growth zone chondrocytes. The control showed a steady level of activity at all inhibitor concentrations. The $1\alpha,25(OH)_2D_3$ treated cells showed significantly higher levels of activity (compared to control) at all inhibitor concentrations. The activity gradually increased with increasing H-89 concentration (non-significant). All values are the mean ± SEM for n=6 cultures. #P<0.05 vs. no treatment with $1\alpha,25(OH)_2D_3$.

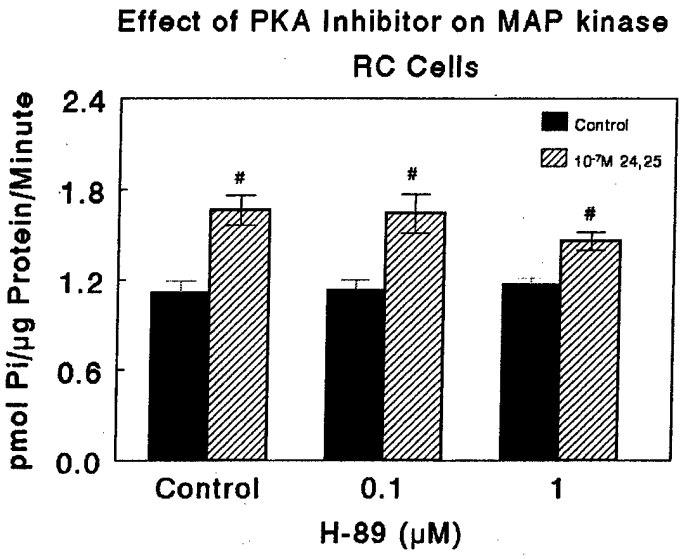


Figure 21: The effect of PKA inhibitor H-89 on MAP kinase specific activity in resting zone chondrocytes. The control showed a steady level of activity at all inhibitor concentrations. The 24R,25$(OH)_2D_3$ treated cells showed significantly higher levels of activity (compared to control) at all inhibitor concentrations. The activity gradually decreased with increasing H-89 concentration (non-significant). All values are the mean ± SEM for n=6 cultures. #P<0.05 vs. no treatment with 24R,25$(OH)_2D_3$.

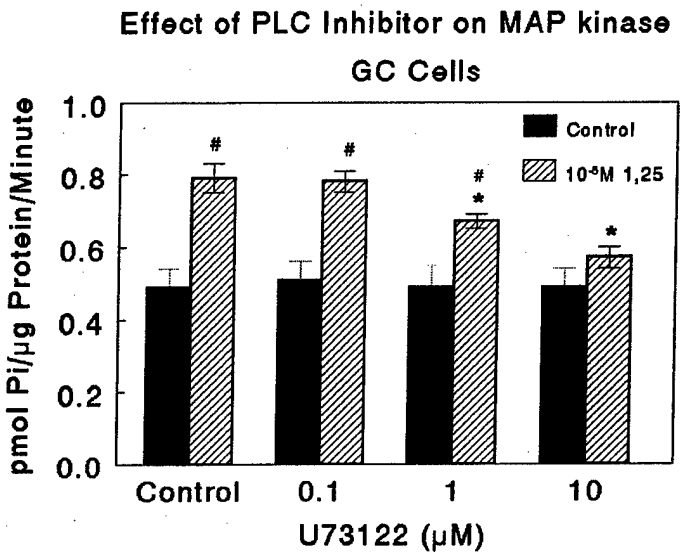


Figure 22: The effect of PLC inhibitor U73122 on MAP kinase specific activity in growth zone chondrocytes. The control showed a steady level of activity at all inhibitor concentrations. The $1\alpha,25(OH)_2D_3$ treated cells showed significantly higher levels of activity at inhibitor concentrations of 0, 0.1, and 1 µM (compared to control). The activity gradually decreased with increasing U-73122 concentration, significantly at 1 and 10 µM. All values are the mean ± SEM for n=6 cultures. *$P<0.05$ vs. no treatment with U73122; #$P<0.05$ vs. no treatment with $1\alpha,25(OH)_2D_3$.

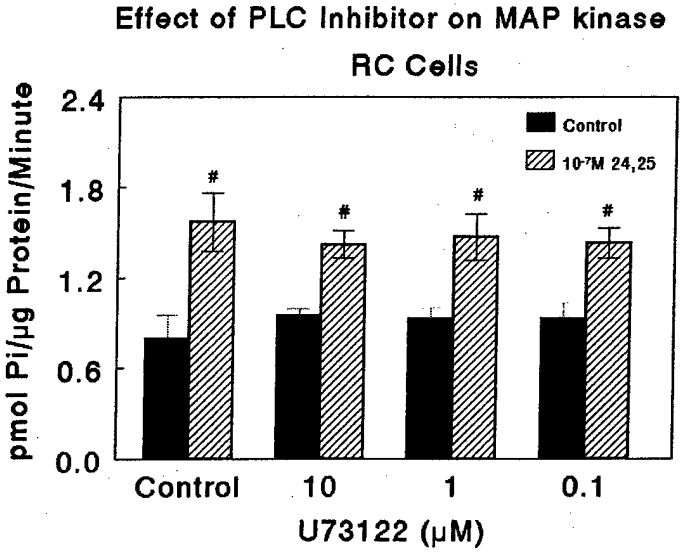


Figure 23: The effect of PLC inhibitor U73122 on MAP kinase specific activity in resting zone chondrocytes. The control showed a steady level of activity at all inhibitor concentrations, which was slightly higher than that at 0 µM U73122. The 24R,25$(OH)_2D_3$ treated cells showed significantly higher levels of activity (compared to control) at all inhibitor concentrations, and the activity remained relatively constant at all U73122 concentrations. All values are the mean ± SEM for n=6 cultures. #P<0.05 vs. no treatment with 24R,25$(OH)_2D_3$.

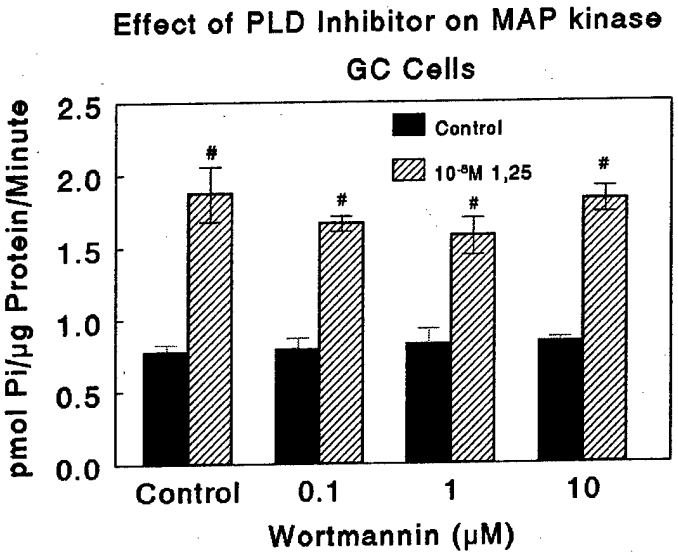


Figure 24: The effect of PLD inhibitor wortmannin on MAP kinase specific activity in growth zone chondrocytes. The control showed steady activity at all inhibitor concentrations. The $1\alpha,25(OH)_2D_3$ treated cells showed significantly higher levels of activity (compared to control) at all inhibitor concentrations. The PLD activity in GC was not affected by wortmannin. All values are the mean ± SEM for n=6 cultures. #$P<0.05$ vs. no treatment with $1\alpha,25(OH)_2D_3$.

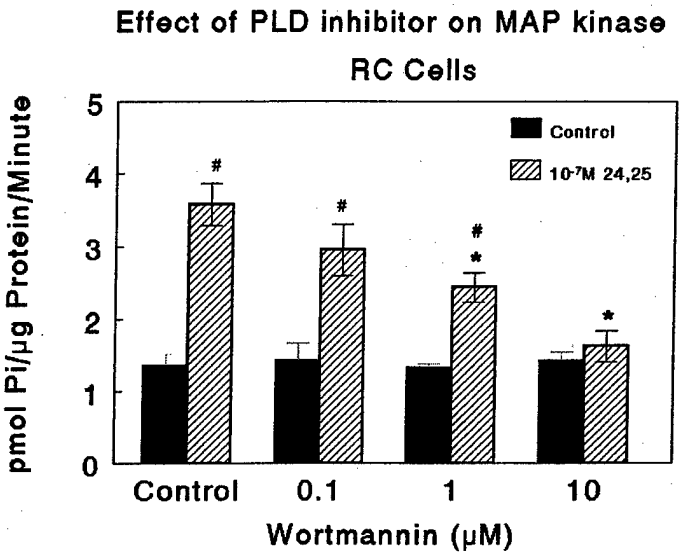


Figure 25: The effect of PLD inhibitor wortmannin on MAP kinase specific activity in resting zone chondrocytes. The control showed a steady level of activity at all inhibitor concentrations. The 24R,25$(OH)_2D_3$ treated cells showed significantly higher levels of activity at inhibitor concentrations of 0, 0.1, and 1 µM (compared to control).The activity steadily decreased as wortmannin concentration increased, and the change was significant at 1 and 10 µM. . All values are the mean ± SEM for n=6 cultures. *P<0.05 vs. no treatment with wortmannin; #P<0.05 vs. no treatment with 24R,25$(OH)_2D_3$.

same extent as seen in cultures treated with 10^{-7} M 24R,25$(OH)_2D_3$. When resting zone cells were treated with indomethacin and 24R,25$(OH)_2D_3$ together, MAP kinase activity was increased by 20% over the amount of activity found in cultures treated with either agent alone (Table 1).

Table 1: Effect of indomethacin on MAP kinase specific activity of growth zone and resting zone chondrocytes treated with vitamin D_3 metabolites.

Growth Zone Chondrocytes	**MAP Kinase (pMol Pi/μg protein/min)**
Control	0.82 ± 0.04
+ Indo (10^{-8} M)	0.69 ± 0.07
+ Indo (10^{-7} M)	0.57 ± 0.09*
1α,25(OH)$_2$D$_3$ (10^{-8} M)	1.24 ± 0.08*
+ Indo (10^{-8} M)	1.06 ± 0.06*•
+ Indo (10^{-7} M)	0.87 ± 0.08*•
Resting Zone Chondrocytes	
Control	1.45 ± 0.15
+ Indo (10^{-8} M)	1.44 ± 0.10
+ Indo (10^{-7} M)	2.10 ± 0.17*#
24R,25(OH)$_2$D$_3$ (10^{-7} M)	2.10 ± 0.11*
+ Indo (10^{-8} M)	2.32 ± 0.13*
+ Indo (10^{-7} M)	2.53 ± 0.22*•

Growth zone cells were treated for 9 minutes with control media or media containing 10^{-8} M 1α,25(OH)$_2$D$_3$ in the presence/absence of the general cyclooxygenase inhibitor indomethacin (Indo). Resting zone cells were treated for 90 minutes with control media or media containing 10^{-7} M 24R,25(OH)$_2$D$_3$ in the presence/absence of Indo. At harvest, MAP kinase activity was determined. All values are the mean ± SEM of n = 6 independent cultures from one experiment. The experiment was repeated twice with nearly identical results. *P<0.05, vs. control; #P<0.05, vs. 10^{-8} M Indo; •P<0.05, vs. 10^{-7} M 24R,25 or 10^{-8} M 1α,25.

DISCUSSION AND SUMMARY

Vitamin D_3 metabolites are essential for chondrocyte differentiation and maturation and are therefore necessary for proper endochondral ossification. MAP kinase is an important mediator of vitamin D_3 action in these cells and is involved in the sequence of phosphorylations that link extracellular signals to intracellular responses. The focus of this study was to examine the role of MAP kinase in the mechanism of action of vitamin D_3 metabolites in resting zone and growth zone chondrocytes. The results demonstrated that ERK-1 and ERK-2 MAP kinases are regulated by vitamin D_3 metabolites in growth plate chondrocytes. This regulation is cell maturation specific: $1\alpha,25(OH)_2D_3$ stimulated MAP kinase activity only in growth zone cells, and $24R,25(OH)_2D_3$ stimulated activity only in resting zone cells.

The vitamin D_3 metabolites activate MAP kinase by phosphorylation rather than a change in mRNA levels or protein synthesis. The Northern blot analysis did not demonstrate differences in mRNA levels for either ERK-1 or ERK-2 MAP kinase in control cultures or $1\alpha,25(OH)_2D_3$- or $24R,25(OH)_2D_3$-treated cultures. Additionally, there were no differences in the amounts of ERK-1 or ERK-2 on Western blots, and enzyme activity was not affected by cycloheximide or actinomycin D in the control or treated cultures. In contrast, MAP kinase specific activity was increased in dose- and time-dependent manners by $1\alpha,25(OH)_2D_3$ in growth zone cells and $24R,25(OH)_2D_3$ in resting zone cells.

The role of protein kinase C in MAP kinase activation was demonstrated with two different PKC inhibitors, chelerythrine and H-7. Results for both inhibitors were similar: the increased MAP kinase activity due to the treatments with vitamin D_3 metabolites was

reduced in a dose-dependent manner. The $1\alpha,25(OH)_2D_3$-dependent stimulation of PKC activity is regulated by two distinct phospholipase-dependent mechanisms: production of DAG, primarily via phospholipase C, and production of arachidonic acid via phospholipase A_2 (Sylvia et al., 1998). The rapid increases in PKC in response to $1\alpha,25(OH)_2D_3$ are mediated, at least in part, via a specific membrane receptor for the vitamin D_3 metabolite (Nemere et al., 1998).

Protein kinase A apparently does not have a role in MAP kinase activation by $1\alpha,25(OH)_2D_3$ or $24R,25(OH)_2D_3$ in growth zone or resting zone chondrocytes, respectively. The PKA inhibitor H-89 had no effect on the control or treated cultures.

Phospholipase C had a cell maturation-dependent effect on MAP kinase activity. The PLC inhibitor U73122 had no effect on growth zone control cultures, but a dose dependent effect on the $1\alpha,25(OH)_2D_3$ treated cultures. In contrast, the U73122 had no effect on the control or treated resting zone cultures.

Phospholipase D had the opposite effect. The PLD inhibitor wortmannin had no effect on MAP kinase activity in treated or control growth zone cultures, but the effect of $24R,25(OH)_2D_3$ treatment in resting zone culture was reduced in a significantly dose-dependent manner.

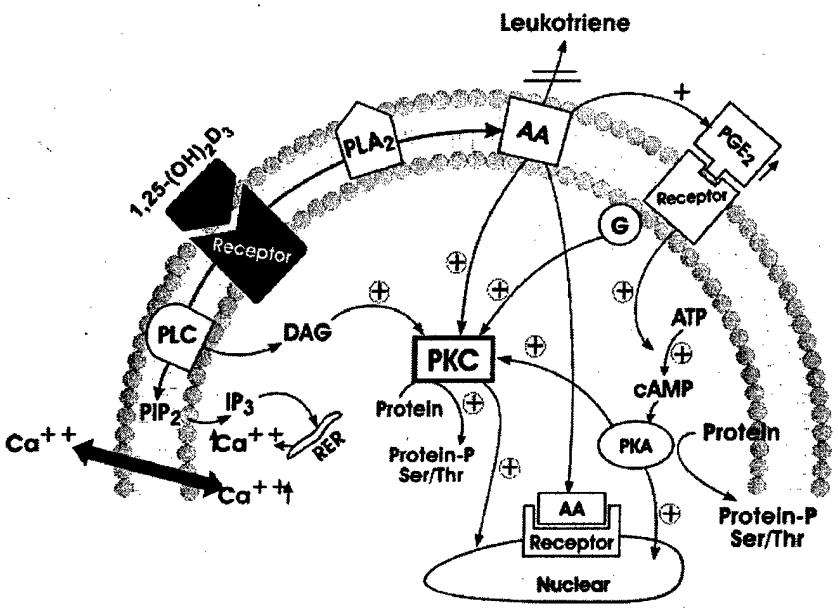
Indomethacin, a cyclooxygenase inhibitor, affected MAP kinase activity in a maturation-dependent manner. In growth zone cultures, indomethacin reduced the enzyme activity in the control and partially reduced the effect due to $1\alpha,25(OH)_2D_3$ treatment. However, in resting zone cultures indomethacin increased MAP kinase activity in the control to the same degree as 10^{-7} M $24R,25(OH)_2D_3$ treatment. The effect of

indomethacin and 24R,25$(OH)_2D_3$ together was greater than the effect of either agent alone.

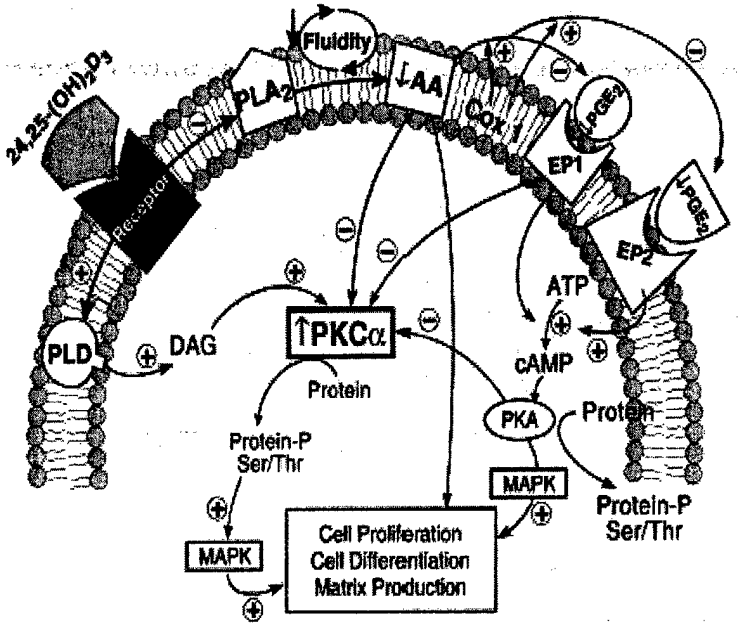
MAP kinase plays different roles in the two growth plate populations with respect to their responses to the vitamin D_3 metabolites. Alkaline phosphatase activity is stimulated by 1α,25$(OH)_2D_3$ in growth zone chondrocytes, and by 24R,25$(OH)_2D_3$ in resting zone chondrocytes (Schwartz et al., 1988c). The MAP kinase inhibitor PD98059 had no effect on alkaline phosphatase activity in either culture following treatment with 1α,25$(OH)_2D_3$ or 24R,25$(OH)_2D_3$. Since 1α,25$(OH)_2D_3$ increases alkaline phosphatase activity in growth zone cell via the 1,25-mVDR and PKC, and 24R,25$(OH)_2D_3$ does the same via the 24,25-mVDR and PKC (Pedrozo et al., 1999), this demonstrated that ERK-1 and ERK-2 were not involved.

The effect on proteoglycan sulfation was quite different. 1α,25$(OH)_2D_3$ causes an increase in [^{35}S]-sulfate incorporation in growth zone chondrocyte cultures, whereas 24R,25$(OH)_2D_3$ regulates proteoglycan sulfation in cultures of resting zone cells (Schwartz et al., 1995). The results demonstrated that ERK-1 and ERK-2 were not involved in proteoglycan synthesis in the growth zone cultures, but did play a role in the resting zone cells.

The proposed mechanisms involving the vitamin D_3 metabolites in the growth zone and resting zone chondrocytes are illustrated in Figure 26. In the growth zone chondrocyte, the 1α,25$(OH)_2D_3$ membrane receptor mediates cell response through activation of PI-PLC, which results in formation of diacylglycerol and inositol-1,4,5-trisphosphate, and leads to activation of PKCα. 1α,25$(OH)_2D_3$ also activates PLA_2, resulting in the release of arachidonic acid, which can activate PKCα. Arachidonic acid



Growth Zone Chondrocytes



Resting Zone Chondrocytes

can also serve as a substrate for cyclooxygenase-1 and result in prostaglandin E_2 (PGE_2) production. PGE_2 can act through its receptor and activate PKA. The signal transduction pathways mediated by PKC and PKA converge at the activation of MAP kinase to modulate gene expression. In the resting zone chondrocyte, the $24R,25(OH)_2D_3$ membrane receptor mediates cell response through activation of PLD, which results in formation of diacylglycerol, and leads to activation of PKCα. $1\alpha,25(OH)_2D_3$ inhibits PLA_2, resulting in a decrease in arachidonic acid, which inhibits PKCα specific activity. The reduced arachidonic acid results in decreased prostaglandin E_2 (PGE_2) production. The PGE_2 acts through its EP1 and EP2 receptors and either inhibit PKC or activate PKA. As in growth zone cells, the signal transduction pathways mediated by PKC and PKA converge at the activation of MAP kinase to modulate gene expression

To summarize, this study supports the hypothesis that MAP kinase mediates the effects of vitamin D_3 metabolites on growth plate chondrocytes. Some of the response of growth zone chondrocytes to $1\alpha,25(OH)_2D_3$ is via the ERK family of MAP kinase, involving rapid PKC-dependent phosphorylation of ERK-1 and ERK-2. $24R,25(OH)_2D_3$ exerts its effects on resting zone chondrocytes in a similar manner. $1\alpha,25(OH)_2D_3$ increases MAP kinase activity via PLC and increased prostaglandin production, while $24R,25(OH)_2D_3$ increases MAP kinase activity via PLD and decreased prostaglandin production. PKA is not involved in the activation of ERK-1 and ERK-2 in either cell type. The stereospecificity of each metabolite, the cell specificity of the effects, and the dependence upon PKC suggest the involvement of membrane receptors for $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ in the regulation of MAP kinase in growth plate chondrocytes.

These results provide another step toward understanding the mechanisms of signal transduction in chondrocytes and osteoblasts, and may lead to new clinical applications involving bone and cartilage formation, repair, and regeneration. This topic is of relevance to dentistry in general, and periodontics in particular, since it involves the regulation of periodontal preservation and regeneration, fracture repair, and dental implant osseointegration.

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VITA

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